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# Differential Protein Expression in Explanted Human Retinal Pigment Epithelial Cells 24-hours Post-Exposure to 532 nm, 3.0 ns Pulsed Laser Light.

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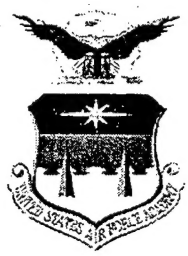

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The use of laser light for targeting devices and weapons has dramatically increased the likelihood that personnel will be exposed to laser energy during military operations. Expanded medical, research, and industrial laser use may lead to excessive risk of exposure of researchers and technicians and also during commercial applications. Further, the nature and importance of the biophysical mechanisms of photon-tissue interaction at such pulse widths and irradiances are not understood at the fundamental cell and molecular level. A human in vitro model for assessing laser-light damage to tissue at the cell and molecular level is desirable for scientific, political and fiduciary reasons. This research assesses the effects of sublethal pulsed laser-light treatment to a human cadaver donor retinal pigment epithelial (RPE) tissue using Western Array screening service with antibodies for 859 known human proteins involved in cell signaling, apoptosis, cell adhesion, kinase and GTPase activity as well as several other functions. Some of these functions have been shown to be important in biological processes that could lead to important biological sequelae such as loss of function, cancer or cell death.This research was done to gain insight into the state of cells at the level of protein expression after laser exposure. To our knowledge this is the first ever application of Western Array screening to laser-bioeffects research.

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Differential Protein Expression in Explanted Human Retinal Pigment Epithelial Cells 24-hours Post-Exposure to 532 nm, 3.0 ns Pulsed Laser Light.

## ABSTRACT

The use of laser light for targeting devices and weapons has dramatically increased the likelihood that personnel will be exposed to laser energy during military operations. Expanded medical, research, and industrial laser use may lead to excessive risk of exposure of researchers and technicians and also during commercial applications. The increased potential for exposure of humans to lasers, especially sub-nanosecond laser pulses in the visible and near-infrared regions of the spectrum (Rockwell, 1999a, b), highlight the need for scientifically based safety standards for laser exposure at the ultra short pulse lengths. The great peak powers achieved at ultrashort pulse lengths suggests that the current standards may not be appropriate to protect personnel. Further, the nature and relative importance of the biophysical mechanisms of photon-tissue interaction at such pulse widths and irradiances are not understood at the fundamental cell and molecular level. Current ultrashort pulse laser safety standards are based on a minimal visible lesion (MVL), i.e. histological, damage endpoint in the Rhesus monkey model (Shaver, 2000). A human *in vitro* model for assessing laser-light damage to tissue at the cell and molecular level is desirable for scientific, political and fiduciary reasons. This research assesses the effects of sublethal pulsed laser-light treatment to a human cadaver donor retinal pigment epithelial (RPE) tissue using BD Transduction Laboratories' BD PowerBlot™ Western Array screening service with antibodies for 859 known human proteins involved in cell signaling, apoptosis, cell adhesion, kinase and GTPase activity as well as several other functions. Some of these functions have been shown to be important in biological processes that could lead to important biological sequelae such as loss of function, cancer or cell death. The results of Western Array immuno-screening analysis of lased human RPE are discussed. This research was done in an effort gain basic scientific insight into the physiological state of cells at the level of protein expression after laser exposure. Of specific interest is the amount and type of damage/perturbation cells undergo due to laser irradiation. To our knowledge this is the first ever application of Western Array screening to laser-bioeffects research.

## BACKGROUND

A search of the literature revealed no other efforts to understand laser-tissue interactions using a proteomic approach. The only other similar work previously performed by Obringer using two-dimensional SDS-PAGE to separate proteins and matrix-assisted laser desorption/ionization mass spectroscopy (MALDI Mass Spec) to

identify proteins of interest in laser exposed ocular cells which laid the basis for the plausibility of this project. Therefore, this research opens a novel approach to investigation of laser-tissue interactions. New aspects include the focus optical tissue explants obtained from human cadaver donors, the use of an *ex vivo* model, and the focus of the investigation at the cell-molecular level using proteomics. Due largely to technical limitations, previous research has focused on laser-tissue interactions at the histological, i.e. tissue level. Most often this has been in corneal and retinal tissues, though there has been some work on dermal tissues (Roach, 2001; Stuck, 1981; Lund, 1970; Zuclich, 1995).

A large void currently exists in the current understanding of laser-tissue interactions at the cell and molecular levels. Specifically, previous research has focused on the (macroscopic) histopathological minimum visible lesion (MVL) threshold of various laser exposures in consideration of multiple variables (Cain, 1995, 1999) while failing to consider possible long-term sequelae of sublethal (non-lesion forming) irradiation or as occurs to the cells in the margins of lesions. Biochemical studies have investigated free radical formation in the ocular melanosomes in retinal pigmented epithelia (pigmented cells behind the retina) which are hypothesized to lead to oxidative damage as one mechanism capable of producing sublethal sequelae of biological significance (Glickman et al., 1996a, 1996b, 1995, 1993, 1992, 1989, Lam et al., 1992).

Laser-tissue research in general is complicated by the number of variables involved, many of which concern technical aspects of laser illumination and the biological model used. In the 1990s an extensive effort was made to revise laser safety standards. This effort examined histological effects as a function of numerous variables, such as wavelength, pulse duration, total number of pulses, time between pulses etc. so that "the list of important variables expands to create a large matrix of possible experimental conditions" (Thomas, 2001). Due to technological limitations however, no attempt was made to undertake a comprehensive examination of the effects of irradiation at the molecular level and develop a similar matrix.

The research described here attempts to gain a preliminary understanding of some details of laser-tissue interactions at the biomolecular (protein) level of cellular physiology. Nonetheless, detailed research at the histological level is not only relevant but also necessary to a full understanding of laser-tissue interactions. As has been noted, this research was conducted on monolayer tissue cultures. While tissue cultures may serve as a model, application of the knowledge gained will be largely in the context of living organisms with fully developed tissues and organs. Thus previous research sets not only the background but also the very foundation for understanding how laser light interacts with the RPE of living persons. We believe that the *ex vivo* explanted tissue model most closely approximates the biological response of the tissue while in the organism.

See USAFA-TR-2004-01 (Obringer, et al., 2004) for further background information on laser-light interaction on optical tissue and USAFA-TR-2002-02 (Lykins, et al., 2002) for background on the mechanisms of physical interaction of laser-light with biological materials.

Western Blotting as a Means of Biological Inquiry

Proteins carry out the majority of chemical activity necessary to life cellular life, serving as structural members of cells, enzymes, and regulating life processes. Western blotting and subsequent immunologic identification of proteins is one method of assaying the proteomic (comprehensive protein) response of a biological system to an environmental stimulus (Kiechle, 2002).

The central dogma of modern biology is that all the chemical/biological information required by a life form is stored in DNA, which is transcribed to RNA and then translated to protein. According to this paradigm the potential response of a biological system to any variation in its environment is encoded in the DNA. The response to an environmental stimulus, such as exposure to a laser beam, may require changes in the transcriptional and translational levels to mediate the required response. Monitoring changes in the transcription levels of various messenger RNAs and the corresponding translation of the proteins they code for would therefore give direct indications of the cell's specific response to the environmental stimulus at the molecular level. Monitoring of protein expression patterns can be accomplished by western blotting techniques in conjunction with immunologic identification of the proteins and bioinformatics analysis of the resultant data set.

Ideally there would be a quantitative means of measuring the transcription, translation and residency times for RNA transcripts and the proteins they code for. In lieu of such a robust system, multiple parallel western blots of cellular extracts are used in this research to query the biological response to laser exposure at the level of translation. By examining the differential protein expression patterns it is expected that insight in to the physiological state of the cells can be ascertained, including but not limited to the amount and type of damage/perturbation the cell has undergone. If this is possible, it may further be possible to predict whether the damage has induced an increased likelihood of future biological perturbation such as mitotic misregulation (an alteration of the control of cellular replication) due to mutagenesis (causing a mutation in the DNA) or a change that will result in the cell undergoing programmed cell death (apoptosis) outside of its normal developmental program.

In this research the BD PowerBlotTM system, of BD Transduction Laboratories, San Diago, CA, was employed to provide proteomic analysis of laser-irradiatedRPE explants. The BD PowerBlot system assays proteins against 859 monoclonal mouse antibodies individually specific for physiologically relevant proteins known to be mediators of processes such as apoptosis, cell adhesion, transcription and translation regulation, cell cycling control and machinery, membrane interaction, organelle function, cytoskeleton components and regulation, nuclear transport, etc. Protein isolated from control versus experimental cells is densiometrically analyzed after contact with the array and compared for significant differences. These differences are presumably indicative of the metabolic differences between lased and unlased cellular responses, and equate to the cell's response at the protein level to laser-induced alteration in cellular physiologic state. Relative changes in expression are verified by comparison of control (sham-exposed) and experimental lysate samples on the same gel.

## MATERIALS AND METHODS

This particular experiment was labeled "N2" (3.0 nanosecond pulse width, 532 nm laser light exposure).

Donor:

The RPE tissue donor for N2 was a 65 year old Caucasian, blue eyed, male that died of cancer. No ocular pathologies were noted.

Explant preparation: See USAFA-TR-2004-01

Laser: Equipment Used for N2

| | |
|---|---|
| Laser (Nd:YAG) | Coherent Infinity XPO Laser |
| Power Meter | Scientech Power Meter model S310 |
| Detector Head | Scientech model PHDX50 |
| Shutter | nmLaser model LS055S3W8 |
| Shutter Controller | nmLaser model CX2450 |
| Velmex XY Stage | model NF90-2 |

Exposures pulse energy was determined by placing a power meter on the x-y translation stage (the site of target exposure) and dividing the measured average power by the pulse repetition rate. This method was considered adequate since pulse-to-pulse energy typically varied less than 10%. The beam profile is a "top hat" with less than 5% variation across the wave front.

Laser-light exposure:

For procedures see USAFA-TR-2004-01. The table below contains the exposure parameters for the experiment reported herein.

| Treatment | **N2** |
|---|---|
| Wavelength (nm) | **532** |
| Average Power (mW) | **508** |
| Pulse Energy (mJ) | **50.8 ± 1.2** |
| Pulse Length (FWHM) | **3.0 ns** |
| Total Incident Energy (mJ) | **3251** |
| Peak Power (W) | **$1.69 \times 10^7$** |
| Fluence (mJ/cm²) | **108** |

| | |
|---|---|
| Exposure Time (sec) | **6.4** |
| Laser Repetition Rate (Hz) | **10** |
| Beam Diameter ($1/e^2$) | **6 mm** |
| Irradiance ($kW/m^2$) | **18.0** |

Total incident energy (TIE) is defined as the amount of laser-light energy that was delivered to the 6 mm well containing the RPE explants. Abbreviations: nm-nanometer; m-meter, mm-millimeter, ns-nanosecond; mJ-milliJoule; mW-milliWatt; FWHM-Full Width Half Max; Hz-Hertz; sec-second; W-watt; e-natural log.

Laser exposure of Human RPE Explants

The Nd:YAG laser light exposure regimen was based on empirical data (not shown) that established cell viability after a range of laser exposures. The exposure described above for treatment N2 was calculated to be 1.8 k $J/m^2$ which is about 10% of the MVL value and approximately 65% above the MPE for the pulse width and wavelength considered (Sliney and Wolbarsht, 1980 and ANSI Z136.1-2000 Table 5a).

In experiment N2 the cells were exposed to either 1) sham exposed to no laser-light (beam blocked upstream), or 2) 64 pulses of 532 nm visible laser-light. Each pulse containing 50.8 mJ ± 1.2 mJ (on average) of energy was delivered to a microtiter plate well 6 mm in diameter containing 50 microliters of medium. See Figure 2 in USAFA-TR-2004-01 for a general overview of the experimental procedures.

Exposed RPE collection

Sample N2 was harvested 24 hours post-exposure for Affymetrix gene chip analysis and protein analysis. A "C" beginning the sample designation (i.e. CN2) indicates the control sample for comparison. The "HX" designation indicates the use of human explanted tissue as the experimental model. See USAFA-TR-2004-01 for further procedural details.

Oligonucleotide Microarray Description Protocol and Analysis:

See USAFA-TR-2004-01 for the oligonucleotide microarray (gene chip) gene expression results.

For a complete listing of sequence sources and human array design the reader should visit Affymetrix's website at www.affymetrix.com, Technical Note: Array Design for the GeneChip Human Genome U133 Set. Figures 3, 4 and 5 of USAFA-TR-2004-01 are offered to familiarize the reader with the Affymetrix platform, general procedures for target preparation and the GeneChip array, respectively.

## Control Procedures

Explants cultured as controls were exposed to the same conditions including transfer to the laser room, exposure to open air for the shoot period, and movement on the x-y translation state in order to expose both samples and controls to the same open air conditions, noise, and electromagnetic fields, etc. The controls, however, were not exposed to laser light, as the laser beam was blocked for the period of time the controls were sham exposed to the open air x-y translation stage conditions.

## Harvest and Cell Lysing for BD PowerBlot™ Assay

Twenty four hours post-treatment, the harvest protocol was initiated. The tissue was placed in labeled, pre-chilled microcentrifuge tubes and stored at -65°C until they were packed on dry ice for overnight express shipment to BD Transduction Labs. Below is a brief overview of the protocol used by the vendor to extract total cellular protein. The harvest protocol consisted of washing twice with Hanks' balanced saline solution (HBSS), once with 50 phosphate-buffered saline (PBS) to remove extraneous protein. The lysis buffer consisting of 10mM Tris (pH 7.4), 1 mM sodium ortho-vanadate, and 1% SDS. The tissue was placed in the lysis buffer and was heated to just below boiling. The lysate was heated in a boiling water bath for 30 seconds and then cellular DNA was mechanically sheared via homogenization. The homogenized samples were then processed as outlined below for BD PowerBlot™ analysis.

## BD PowerBlot™ Materials and Methods

The following procedures were performed by BD Transduction Laboratories, 133 Venture Ct.Lexington, Kentucky 40511. Descriptions of procedures conveyed below were provided by Ms. Bryden Heywood at BD Transduction Labs.

Experimental and control samples were received, thawed and total protein quantitated using the Pierce BCA reagent colorimetric assay to ensure adequate sample quantity. Protein concentration in solution was equalized by dilution and the samples are loaded for SDS PAGE. The separated proteins on the PAGE gels were transferred to PVDF membranes (western blotted) so that samples of the proteins were transferred to the surface of the membrane, replicating their exact relative positions on the PVDF membrane. This allows correlation of lane number and molecular weights (as determined by electrophoresis) with protein identification obtained from the next steps. The membranes containing the electrophoretically separated proteins are then probed with specific mouse monoclonal antibodies. The complete list of these antibodies can be found in Appendix A. Antibodies bind to those proteins in the blots for which they are specific, while antibodies not bound are rinsed away. The antibody-protein complexes are then incubated with fluorescently tagged anti-mouse antibodies. Binding of the fluorescently tagged antibodies allows the bound proteins to be visualized under the proper wavelength of light. The data is captured as an electronic photograph when membranes are exposed to infrared light and read with the Odyssey™ Infrared Imaging System. PDQuest software (by Bio-Rad) automates the spot-finding and spot-matching functions that correlate SDS Page information (molecular weight) with Western blot data

(immunologic binding, spot size). Finally, bioinformatics techniques are used to generate confidence levels based on reproducibility, fold change and spot intensity, and present the data set in an MS Excel spreadsheet (see Appendix A).

The Western blotting protocol is listed below, per the BD Transduction Labs PowerBlot™ Service product information sheet, (Bryden Heywood, January 2002, personal communication). Prior to this procedure protein concentrations were quantitated using the Pierce BCA reagent colorimetric assay. Protein concentrations were then equalized by dilution and rechecked prior to Western blotting as described below.

Western blotting

1. The gel is 13x1 0 cm, 4-15% gradient SDS-polyacrylamide, 0.5mm thick (Bio-Rad Criterion IPG well comb). A gradient system is used so a wide size range of proteins can be detected on one gel.
2. 200 ug of protein is loaded in one big well across the entire width of the gel. This translates into -10 ug per lane on a standard 10 well mini-gel. The gel is run for 1.5 hours at 150 volts.
    1. The gel is transferred to Immobilon-P membrane (Millipore) for 2 hours at 200 mAmp. We use a wet electrophoretic transfer apparatus TE Series from Hoefer.
    2. After transfer, the membrane is dried and re-wet in methanol. The membrane is blocked for one hour with blocking buffer (LI-COR).
    3. Next, the membrane is clamped with a western blotting manifold that isolates 40 channels across the membrane. In each channel, a complex antibody cocktail is added and allowed to hybridize for one hour at 372C.
    4. The blot is removed from the manifold, washed and hybridized for 30 minutes at 372C with secondary goat anti-mouse conjugated to Alexa680 fluorescent dye (Molecular Probes).
    5. The membrane is washed, dried and scanned using the Odyssey Infrared Imaging System (LI-COR).
6. MW Standards - Standards are composed of an antibody cocktail added to lane 40 of Template A, B, C, and D PowerBlot gels. Lanes 16 and 17 of Template E blots are loaded with two standard cocktails, standard cocktail #1 and standard cocktail #2, respectively.

MW Standards: (kD)

| | |
|---|---|
| P190 Glued | 190 |
| Adaptin beta | 160 |
| STAT-3 | 92 |
| Mek-2 | 46 |
| RACK-1 | 36 |
| GRB-2 | 24 |
| Rap2 | 21 |

Standard Cocktail#1: (kD)

| | |
|---|---|
| Exportin-1/CRM1 | 112 |
| MCM | 83 |
| Nucleoporin p62 | 62 |
| a-tubulin | 55 |
| Actin | 42 |
| KNP-1/HES1 | 28 |
| NTF2 | 15 |

Standard Cocktail#2: (kD)

| | |
|---|---|
| p190 | 190 |
| Hip1R | 120 |
| Transportin | 101 |
| Calreticulin | 60 |
| Arp3 | 50 |
| e1F-6 | 27 |
| Rap2 | 21 |

Statistical Procedures

The following information was provided by BD Transduction Laboratories, Lexington, Kentucky, regarding statistical analysis of samples and interpretation of resulting data labels: Fold change is a "semiquantitiave value" representing the general trend of protein changes for the experimental sample relative to the control. Protein expression levels were determined by the following procedure: Separated proteins from SDS-PAGE are transferred to PVDF membranes by Western Blot, assayed with cocktails of mouse-derived antibody cocktails (listed above), and rinsed. The mouse antibodies are then incubated with fluorescently tagged anti-mouse antibodies. Binding of the fluorescently tagged antibodies allows the bound proteins to be visualized under the proper wavelength of light. The data is captured as an electronic photograph when the membranes are exposed to infrared light and read with the Odyssey™ Infrared Imaging System. PDQuest software (by Bio-Rad) automates the spot-finding and spot-matching functions that correlate SDS Page information (molecular weight) with Western blot data (immunologic binding, spot size). Raw data was the total intensity value of a spot.

Calculation of Fold Change

The raw values determined as described above were normalized by dividing the raw quantity of a spot by the total intensity value of all pixels in an image multiplied by 1,000,000. The ratio of normalized quantity for experimental samples to the corresponding spot on the control sample is the protein expression fold change. Specifically, differential protein expression, i.e. "fold change" was calculated by dividing the signal strength of the experimental sample by that of the control sample for each run

of the Western blots. "+" Indicates an increase in expression of a protein in the laser exposed samples, while "-" indicates a decrease in expression, and div/0 indicates that the protein was not detected in the experimental but was detected in the controls or it was detected in the control sample but not the experimental sample, so that calculation of fold change would result in irrational number (division by zero). Thus + div/0 means the protein was detected in the laser exposed sample but not the control, while – div/0 indicates the protein was detected in the control but not in the laser-exposed (experimental) sample.

A protein fold expression confidence level was calculated for each protein based on a combination of signal strength and the absolute value of the fold increase itself, as well as whether all Western Blot runs agreed in the direction of fold change (i.e. did they all increase/decrease, or was one run in conflict with the others). Following is a guide to data interpretation of the protein fold changes presented in Appendix A.

Data analysis

Data analysis includes raw and normalized signal intensity data from each blot with changes greater than 1.25 fold indicated. A description of characteristics of the analysis follows immediately:

1. Quantity - total intensity of a defined spot.
2. Normalized Quantity - the raw quantity of a spot divided by the average intensity value of normalization standards in an image.
3. Ratio - The Normalized Quantity for experimental bands expressed as a ratio of the Normalized Quantity for the corresponding control bands. The Ratio is used to determine increases or decreases in protein expression.
4. Samples run in duplicate and analyzed using a 2X2 matrix comparison method. For example, run 1 of control is compared to runs 1 and 2 of experimental. Run 2 of control is compared to runs 1and 2 of experimental.
5. Results are also expressed as Fold Change, a.semi-quantitative value that represents the general trend of protein changes, either increasing or decreasing, for the experimental sample relative to control.
6. Changes are listed in order of confidence, level 10 being the highest confidence. Confidence levels are defined as:
   a) Level 10 - Changes greater than 2 fold in all 4 comparisons from good quality signals that also pass a visual inspection
   b) Level 9 - Changes 1.5 to 1.9 fold in all 4 comparisons from good quality signals that also pass a visual inspection
   c) Level 8 - Changes greater than 2 fold in all 4 comparisons from low signals that pass a visual inspection
   d) Level 7 - Changes 1.25 to 1.5 fold in all 4 comparisons from good quality signals that pass a visual inspection
   e) Level 6 - Changes greater than 2 fold in all 4 comparisons that do not pass visual inspection
   f) Level 5 - Changes 1.5 to 1.9 fold in all 4 comparisons that do not pass visual inspection

g) Level 2 - Changes greater than 2 fold in all 4 comparisons from low signals that do not pass a visual inspection
h) Level 3 - Changes 1.25 to 1.5 fold in all 4 comparisons from good quality signals that do not pass a visual inspection
i) Level 2a - Changes 1.5 to 1.9 fold in all 4 comparisons from low quality signals
j) Level 2a - Changes 1.25 'to 1.5 fold in all 4 comparisons from low quality signals
k) Level 1a - Changes greater than 2 fold in 3 of 4 comparisons from good quality signals
I) Level 1 b - Changes 1.5 to 1.9 fold in 3 of 4 comparisons from good quality signals
m) Level 0 - No significant protein changes

As mentioned, fold changes represented as div/0 in the appendices represent the presence verses absence of a protein, in which case the fold change cannot be calculated mathematically because division by zero is not possible. In essence, these changes represent a profound change in signal strength. Signals with numeric levels below 30,000 as read by the Odyssey™ Infrared Imaging System, were deemed low signals. "Comparisons based on low signals can be inconsistent" (BD PowerBlot™ Service Technical Data Sheet). Technical descriptions of the proteins identified can be found by accessing the PowerBlot Informatics website at **http://bioinfo.clontech.com/powerblot**. From this site the reader can access the online catalog and review the technical data sheets that describe known protein functions and expressions patterns, as well as, link to the Swiss-Prot and Locus-Link protein databases.

## RESULTS

Appendix A contains the results of the BD PowerBlot™ analysis. A total of 104 proteins showed some level of significant change and an additional 17 were identified to be present in measurable quantities, but had not significantly changed from the control. The table below indicates the confidence level with the number of proteins classified in that level and the number that increased (up) or decreased (down) in the order of confidence (10 being the highest).

| Confidence Level | number of proteins | Up | Down |
|---|---|---|---|
| 10 | 7 | 4 | 3 |
| 9 | 4 | 0 | 3 |
| 8 | 1 | 1 | 0 |
| 7 | 6 | 4 | 2 |
| 6 | 0 | 0 | 0 |
| 5 | 7 | 5 | 2 |
| 4 | 13 | 4 | 9 |
| 3 | 21 | 3 | 18 |
| 2a | 15 | 5 | 10 |
| 2b | 14 | 5 | 9 |
| 1a | 3 | 2 | 1 |
| 1b | 13 | 6 | 7 |

| | | | |
|---|---|---|---|
| 0 | 17 | - | - |
| | 104 | 39 | 64 |

Clearly the number of identifiable proteins that decreased in concentration after laser exposure is nearly 2 (1.6) to 1 compared to the number that increased. Also, there were several proteins that were altered in near or greater than 5 fold concentration after laser-light exposure that would most likely qualify as excellent candidates for biomarker investigations. A few were Calreticulin (+), Calnexin 105kD (+), Annexin VI-78kD (+) and P-Cadherin (-) , interestingly all involved in Ca-binding and/or signaling. Also of note were Clatherin Heavy Chain-180kD (+), Gelsolin (+), V-1/Myotropin (+) and EPLIN (-) all involved in cytoskeletal structure and/or metabolism. Obviously, those that increased in concentration after treatment would be the primary candidates for further investigations as biomarkers.

## DISCUSSION

An analysis of the change in protein concentrations tabulated in Appendix A reveals that the RPE cells lased with 3 ns pulsed, 532 nm laser-light and assayed 24 hours post exposure had a number of physiological responses to the treatment. One striking observation was that Calnexin-105 kD increased an average of approximately 6 fold across the 4 comparisons. Calnexin is a calcium-binding protein that interacts with the newly synthesized glycoproteins in the endoplasmic reticulum. It is also reputed to act in assisting protein assembly and/or in the retention within the ER of unassembled protein subunits. It seems to play a major role in the quality control apparatus of the endoplasmic reticulum by the retention of incorrectly folded proteins (Swiss-Prot:P27824). UbcH7 was increased by nearly 3-fold across 3 comparisons as well as UbcH6. These findings are consistent with the previous observations that 120 ps, pulsed 532 nm laser-light disrupts the function of the protein synthesis apparatus and induces the ubiquitin-proteosome pathway (UPP) (Obringer, et al. 2004). The total energies for the two experiments were nearly identical, but the difference between this and the experiment reported in USAFA-TR-2004-01 is the pulse width (3 ns vs 120 ps) thus resulting in a 25-fold increase in peak power [Peak Power (W) $1.69 \times 10^7$ vs Peak Power (W) $4.21 \times 10^8$]. In the picosecond treated RPE the devastation to the protein synthesis apparatus and protein structure appeared to be far more extensive based on gene expression profiling which makes intuitive sense given the difference in peak power. See USAFA TR-2004-02 for the gene expression profile for this experiment which indicates a 5-fold increase in the gene expression of ribosomal protein L37a mRNA, just to highlight one, providing evidence of protein synthetic apparatus perturbation when assayed at the gene expression level. So it would appear that gene expression profiling and protein concentration profiling are graded responses that are concordant with each other as to the physiological consequence in this metabolic pathway.

Further examination of the data in Appendix A reveals that several proteins involved in all involved in Ca-binding and/or signaling. Such as Calreticulin (+), Calnexin 105kD (+), Annexin VI-78kD (+) and P-Cadherin (-) and others differentially expressed at the protein level thus indicating a perturbation to calcium trafficking affecting several cellular/physiological pathways.

Consistent with this same pattern are the observations of alterations in cytoskeletal metabolism with the down regulation of EPLIN and the up regulation of gelsolin, clatherin and E, NCK, Paxillin-109kD and P-cadherin to highlight a few. Overall, the cell appears to be remodeling the microtubecular lattice and plasma membrane adaptors and cytoskeletal-connected membrane associated surface architectures, some dramatically. For example, Caveolin 1-81 kD, a surface adaptor, showed over a 20 fold increase in two of the 4 assays, averaging a 13 fold increase across the 4 assays.

In a few other systems, proteins involved in apoptosis showed a mixed response, but overall indicated a non-apoptotic response. And the affect on cell cycling indicates a slowing or halting of mitosis including DNA replication, nucleotide synthesis, and cell division. Not surprisingly, these findings are consistent with previous work at the gene expression level.

To the best of our knowledge, this report represents the first-ever application of differential protein expression analysis to assess laser-light induced perturbation within the metabolic framework of explanted human cadaver RPE cells. It is heartening to note that the differential protein expression pattern largely agrees with the differential genes expression patterns reported previously. It is our opinion that the melding of the genomics and proteomics of laser tissue-interaction will provide powerful insight into the viable functioning of tissue after an environmental stressor occurrence, whether it be laser-light exposure or some other agent.

## REFERENCES


Cain, C. P., Toth, C. M., Noojin, G. D., Carothers, V., Stolarski, D. J., Rockwell, B. A. Thresholds for visible lesions in the primate eye produced by ultrashort near-infrared laser pulses. *Investigative Ophthalmology and Visual Science.* **40**: 2343-2349. (1999).

Cain, C. P., Toth, C. M, DiCarlo, C. A., Stein, C. D., Noojin, G. D., Stolarski, D. J., et al. Visible retinal lesions from ultrashort laser pulses in the primate eye. *Investigative Ophthalmology and Visual Science.* **36**:879-888 (1995).

Glickman, R. D. A study of oxidative reactions mediated by laser-excited ocular melanin. AL/OETR **12**:1-20 (1996a).

Glickman, R. D., Jacques S. L., Schwartz, J. A., Rodriques T., Lam. K. W., Buhr, G. Photodisruption increases the free radical reactivity of melanosomes isolated from retinal pigment epithelium. *Laser-Tissue Interaction VII.* **2681**:460:467 (1996b).

Glickman, R. D., Lam K. W. Melanin may promote photooxidation of linoleic acid. *Laser-Tissue Interaction VI.* **2391**:254-261 (1995).

Glickman, R. D., Sowell R., Lam K. W. Kinetic properties of light-dependent ascorbic acid oxidation by melanin. *Free Radical Biology & medicine* **15**:453-457 (1993).

Glickman, R. D., Lam K. W. Oxidation of ascorbic acid as an indicator of photooxidative stress in the eye. *Photochem and Photobio* **55**:191-196 (1992).

Kiechle, F. L, Zhang, X. The postgenomic era. *Arch Pathol Lab Med.* **126**(3):255-62 (2002).

Lam, K. W.,Glickman, R. D. Prevention of light-induced free radicals production from melanin granules by ascorbic acid. Oxygen Radicals. 633-636(1992).

Lund, D. J., Landers, M. B., Bresnick, G. H., Powell, J. O., Chester, J. E., Carver, C. Ocular Hazards of the Q-switched Erbium Laser. *Investigative Ophthalmology.* **9**: 463-470 (1970).

Lykins, D. R., Obringer J. W., Johnson M. D. Laser Bioeffects: Differential protein expression of cultured human melanocytes treated with 532 nm picosecond pulse laser-light. Technical Report: USAFA-TR-2002-02 (2002).

Obringer, J. W., Johnson, M. D. Differential gene expression in explanted human retinal pigment epithelial cells 24-hours post-exposure to 532 nm, 3.0 ns pulsed laser-light and 1064 nm, 170 ps pulsed laser-light 12-hours post-exposure:Results compendium. Technical Report: USAFA-TR-2004-02 (2004).

Obringer, J. W., Johnson, M. D. Differential gene expression in explanted human retinal pigment epithelial cells 12-hours post-exposure to 532 nm, 120 ps pulsed laser-light. Technical Report: USAFA-TR-2004-01 (2004).

Roach, W. P., Johnson, T. E. Porcine Dermal Lesions Produced by 1540 nm Laser Radiation Pulses. *Proceedings of SPIE.* **4257**:363-369 (2001).

Rockwell, B. A., Hammer, D. X., Hopkins, R. A., Payne, D. J., Toth, C. A., Roach, W. P., et al. Ultrashort laser pulse bioeffects and safety. *Journal of Laser Applications.* **11**:42-44 (1999a).

Rockwell, B. A., Toth, C. A., Roach, W. P., Payne, D. J., Hopkins, R. A., Kennedy, P. K., et al. Retinal damage mechanisms and safety for ultrashort laser exposure. *Proceedings of SPIE.* **3601**:4-9 (1999b).

Shaver, J. H., Buffington, G. D., Rockwell, B. A., Thomas, R. J., Cain, C. P., Noojin, G. D., Stolarski, D. J. Effects of Dispersion & Aberrations in Ocular Focusing of Femtosecond Pulses & the Impact on Damage Mechanisms. *Laser-Induced Damage in Optical Materials: 2000. SPIE.* **4347**:267-276 (2001).

Sliney, D., Wolbarsht, M. Safety with Lasers and Other Optical Sources, A Comprehensive Handbook.ISBN 0-306-40434-6, Plenum Press:77-85 (1980)

Stuck, B. E., Stuck, D. J., Beatrice, E. S. Ocular effects of laser radiation from 1.06 to 2.06μ. *Health Physics.* **40**:835-846 (1981).

Thomas, R. J., Noojin, G. D., Stolarski, D. J., Hall, R. T., Cain, C. P., Toth, C. A., et al. A comparative study of ocular damage thresholds from continuous-wave and femtosecond mode-locked lasers. *Laser and Noncoherent Light Ocular Effects: Epidemiology, Prevention, and Treatment. Proceedings of SPIE.* **4246**:54-62 (2001).

Zuclich, J. A., Gagliano, D. A., Cheney, F., Stuck, B. E, Zwick, H., Edsall, P. et al. cular Effects of Penetrating IR Laser Wavelengths. *Laser Tissue Interaction VI SPIE.* **2391**

# APPENDIX A

Summary key

1 Fold Change = a semiquantitative value that represents the general trend of protein changes for the experimental sample relative to control
2 "-" = represents a decrease in protein level in the experimental sample relative to control
3 "+" = represents an increase in protein level in the experimental sample relative to control
4 Confidence level = proteins are compared using a 2X2 matrix, R1 of control is compared to R1 and R2 Experimental, Run 2 of control is compared to R1 and R2 of Experimental. Protein changes are listed in order of confidence, 10 being the highest confidence:
Level 10 - Changes greater than 2 fold in all 4 comparisons that are from good quality signals and pass a a visual inspection
Level 9 - Changes 1.5-1.9 fold in all 4 comparisons that are from good quality signals and pass a visual inspection
Level 8 - Changs greater than 2 fold in all 4 comparisons from low quality signals that pass a visual inspection
Level 7 - Changes 1.25-1.5 fold in all 4 comparisons that are from good quality signals and pass a visual inspection
Level 6 - Changes greater than 2 fold in all 4 comparisons that are from good quality signals and do not pass a a visual inspection
Level 5 - Changes 1.5-1.9 fold in all 4 comparisons that are from good quality signals and do not pass a visual inspection
Level 4 - Changs greater than 2 fold in all 4 comparisons from low quality signals that do not pass a visual inspection
Level 3 - Changes 1.25-1.5 fold in all 4 comparisons that are from good quality signals and do not pass a visual inspection
Level 2a - Changes 1.5-1.9 fold in all 4 comparisons from low signals
Level 2b - Changes 1.25-1.5 fold in all 4 comparisons that are from low signals
Level 1a- Changes greater than 2 fold in 3 of the 4 comparisons that are from good quality signals
Level 1b - Changes 1.5-1.9 in 3 of the 4 comparisons that are from good quality signals
Level 0 - No significant changes
5 div/0 = represents presence verses absence of a protein, fold change is unmeasurable
6 ls = low signal, signals with a normalized quantity <500 or low quality, results can be inconsistent.
7 Quantity = raw signal intensity derived from blots loaded with equal protein
8 Norm Qty = normalizes for variations in exposure levels, the raw quantity of a spot is divided by the total intensity value of all valid spots in an image and multiplied by 1,000,000 .
9 proteins having multiple bands have observed molecular weight included in name
10 "SSP" = standard spot number, used to reference spots in analysis software

**BD PowerBlot Obringer Analysis** — **Summary of Changes Samples 1 & 2** — **Changes in 2 relative to 1**

| SSP | Catalog | Template | Lane | Protein ID | Area of Research | Predicted Mol. Wt. | Obs. MW | Low Signal Count | Sign of Change | Fold Change R1/R1 | Fold Change R2/R2 | Fold Change R2/R1 | Fold Change R1/R2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Level 10 - changes greater than 2 fold in 4 of 4 comparisons from good quality signals that pass a visual inspection** | | | | | | | | | | | | | |
| 1905 | C45520 | B | 2 | Calnexin-105KD | Calcium Signa | 90 | 104 | 2 | + | 8.59 | 4.63 | 7.71 | 5.16 |
| 3304 | C64020 | D | 9 | Cathepsin D-27KD | Cancer Resear | 43/28 | 27 | 0 | - | 2.17 | 2.24 | 2.33 | 2.10 |
| 1902 | C43820 | E | 5 | Clathrin Heavy Chain-180KD | Adaptors & Ty | 180 | 180 | 1 | + | 2.77 | 7.46 | 7.20 | 2.87 |
| 4903 | E35520 | B | 12 | EPLIN | Cytoskeleton ( | 90/110 | 122 | 2 | - | 3.38 | 4.79 | 5.00 | 3.24 |
| 1704 | G37820 | D | 2 | Gelsolin | Cancer Resea | 93 | 95 | 0 | + | 7.13 | 2.07 | 4.29 | 3.44 |
| 1909 | C24120 | D | 2 | P-Cadherin | Cancer Resea | 120 | 117 | 2 | - | 5.46 | 3.03 | 5.51 | 3.01 |
| 3603 | P20120 | D | 10 | Sam68 | Adaptors & Ty | 68 | 73 | 0 | + | 3.58 | 2.36 | 2.89 | 2.93 |
| **Level 9 - changes 1.5 to 1.9-fold in 4 of 4 comparisons from good quality signals that pass a visual inspection** | | | | | | | | | | | | | |
| 6702 | R14320 | B | 28 | A-Raf | MAP Kinase P | 68 | 67 | 0 | - | 2.40 | 2.64 | 4.13 | 1.54 |
| 2605 | A27320 | D | 5 | Acetylcholinesterase | Neuroscience | 68 | 73 | 0 | - | 1.69 | 1.65 | 1.72 | 1.61 |
| 5801 | F11020 | B | 19 | FIP-2 | Apoptosis Nuc | 74 | 76 | 0 | - | 2.26 | 1.81 | 2.02 | 2.02 |
| 3002 | R56220 | A | 8 | Rac1-18KD (minor band) | Membrane Res | 21 | 18 | 2 | - | 1.59 | 1.70 | 1.81 | 1.49 |
| **Level 8 - changes greater than 2 fold in 4 of 4 comparisons from low quality signals that pass a visual inspection** | | | | | | | | | | | | | |
| 3601 | A30120 | C | 10 | Annexin VI-78KD | Calcium Signa | 70 | 79 | 3 | + | 4.30 | 2.38 | 4.63 | 2.21 |
| **Level 7 - changes 1.25 to 1.5-fold in 4 of 4 comparisons from good quality signals that pass a visual inspection** | | | | | | | | | | | | | |
| 6501 | A47520 | D | 22 | beta-Arrestin1-54KD | Neuroscience | 55 | 54 | 0 | + | 1.28 | 2.49 | 1.61 | 1.97 |
| 8103 | 680405 | D | 33 | Caspase-6-11KD | 0 | 35/11 | 11 | 2 | - | 1.46 | 1.91 | 1.25 | 2.23 |
| 7301 | M64620 | A | 23 | Caspase-7/MCH-3 | Apoptosis | 35 | 33 | 1 | + | 1.29 | 2.07 | 1.47 | 1.81 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7302 | J36020 | B | 33 | JAM-1-33KD | Cell Adhesion | 38 | 32 | 2 | + | 1.99 | 1.54 | 1.40 | 2.19 |
| 1502 | N15920 | B | 2 | NCK | Adaptors & Ty | 47 | 50 | 1 | + | 6.14 | 1.42 | 3.93 | 2.23 |
| 2302 | T33520 | A | 6 | TIAR-37KD | Apoptosis Nu | 50/42 | 36 | 2 | + | 1.26 | 2.62 | 2.39 | 1.39 |
| **Level 6 - changes greater than 2 fold in 4 of 4 comparisons from good quality signals that do not pass a visual inspection** | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| **Level 5 - changes 1.5 to 1.9-fold in 4 of 4 comparisons from good quality signals that do not pass a visual inspection** | | | | | | | | | | | | | |
| 3605 | A31320 | D | 9 | AKAP79 | Protein Kinas | 79 | 73 | 0 | - | 1.70 | 2.22 | 1.55 | 2.43 |
| 1805 | C43820 | E | 5 | Clathrin Heavy Chain-157KD? | Adaptors & Ty | 180 | 157 | 1 | + | 1.50 | 4.19 | 1.68 | 3.73 |
| 1401 | C12620 | B | 1 | Crk | Adaptors & Ty | 40 | 39 | 0 | - | 1.56 | 1.48 | 1.55 | 1.49 |
| 5701 | I81720 | D | 17 | Integrin beta 3 | Immunology C | 104 | 106 | 2 | - | 2.03 | 1.51 | 1.53 | 2.00 |
| 6503 | S68020 | C | 31 | SHC-49KD | Cell Biology A | 66/52/46 | 49 | 2 | + | 2.13 | 1.74 | 2.12 | 1.75 |
| 3307 | S92220 | D | 11 | Syntaxin 8 | Cell Biology | 27 | 23 | 2 | - | 1.79 | 1.47 | 1.80 | 1.47 |
| 3506 | Z24820 | C | 11 | ZAP70 Kinase | Tyrosine Kina | 70 | 71 | 0 | - | 1.55 | 1.93 | 1.72 | 1.74 |
| **Level 4 - changes greater than 2 fold in 4 of 4 comparisons from low quality signals that do not pass a visual inspection** | | | | | | | | | | | | | |
| 6702 | D27120 | D | 23 | Dynamin II | Adaptors & Ty | 100 | 105 | 4 | - | 2.09 | 2.23 | 2.00 | 2.33 |
| 1505 | F19720 | C | 4 | fyn | Tyrosine Kinas | 59 | 64 | 3 | - | 2.59 | 3.65 | 2.21 | 4.27 |
| 4902 | G10020 | C | 15 | GMAP-210 | Mitochondria/C | 210 | 213 | 4 | + | div/0 | div/0 | div/0 | div/0 |
| 4407 | H22020 | C | 20 | Hsp40 | Cancer Resea | 40 | 40 | 4 | - | 4.23 | 7.20 | 11.15 | 2.73 |
| 2811 | N39120 | E | 8 | iNOS/NOS Type II-141KD | Nitric Oxide | 130 | 142 | 4 | + | div/0 | div/0 | div/0 | div/0 |
| 7402 | J36020 | B | 33 | JAM-1-35KD | Cell Adhesion | 38 | 35 | 4 | - | 3.28 | 5.09 | 5.06 | 3.29 |
| 4401 | P69120 | A | 14 | p47A | Membrane Re | 50/47 | 48 | 4 | - | 2.74 | 3.37 | 3.49 | 2.65 |
| 5802 | P49620 | E | 18 | Paxillin-109KD? | Adaptors & Ty | 68 | 109 | 4 | - | 4.90 | 2.02 | 3.61 | 2.74 |
| 1901 | R27020 | C | 5 | Rb2 | Cell Cycle Car | 130 | 137 | 4 | + | div/0 | div/0 | div/0 | div/0 |
| 4901 | R97120 | D | 14 | RECK | Cancer Resea | 110 | 114 | 4 | - | 2.11 | 12.85 | 11.99 | 2.27 |
| 2808 | R32820 | E | 9 | Rin1-111KD? | GTPases GTP | 90 | 111 | 4 | - | 1.92 | 2.03 | 1.93 | 2.02 |
| 2902 | R32820 | E | 9 | Rin1-178KD? | GTPases GTP | 90 | 178 | 4 | - | 9.45 | 2.04 | 6.48 | 2.98 |
| 7003 | V11220 | D | 31 | V-1/myotrophin | Cell Cycle Ne | 11 | 11 | 3 | + | 7.27 | 2.11 | 3.13 | 4.90 |
| **Level 3 - changes 1.25 to 1.5 fold in 4 of 4 comparisons from good quality signals** | | | | | | | | | | | | | |
| 6201 | A12820 | A | 21 | Acrp30/Adiponectin | Protein Sortin | 30 | 26 | 2 | - | 2.07 | 1.49 | 1.35 | 2.28 |
| 2402 | B33720 | A | 4 | BAF47 | Nucleus & Nu | 47 | 45 | 2 | - | 1.40 | 2.19 | 1.83 | 1.67 |
| 4103 | F67720 | B | 15 | basic FGF-19KD | Cancer Resear | 18-24 | 19 | 0 | - | 1.41 | 1.39 | 1.48 | 1.32 |
| 7304 | B61220 | B | 34 | Bcl-x | Apoptosis | 26 | 27 | 2 | - | 3.02 | 1.53 | 3.53 | 1.31 |
| 8502 | C41720 | B | 38 | Calreticulin | Calcium Signa | 60 | 54 | 1 | + | 6.37 | 1.36 | 3.53 | 2.45 |
| 5102 | C37120 | C | 24 | Caveolin 1 | Adaptors & Ty | 22 | 20 | 1 | - | 2.10 | 1.41 | 1.78 | 1.66 |
| 5901 | C25020 | B | 19 | CHD3-168KD(fragment?) | Immunology N | 221 | 169 | 0 | - | 1.75 | 1.46 | 1.28 | 2.00 |
| 6402 | C14520 | D | 23 | Csk | Tyrosine Kinas | 50 | 45 | 2 | - | 1.39 | 1.61 | 1.26 | 1.77 |
| 4501 | E88120 | B | 13 | ECA39 | Cell Cycle Tra | 43 | 44 | 0 | - | 1.51 | 1.60 | 1.28 | 1.89 |
| 4301 | E80720 | C | 16 | eIF-6 | Cancer Resear | 27 | 24 | 0 | - | 1.54 | 1.27 | 1.39 | 1.40 |
| 9501 | M12320 | E | 40 | ERK1 | MAP Kinase P | 44/42 | 40 | 0 | + | 2.50 | 1.32 | 1.80 | 1.84 |
| 5702 | E53020 | D | 21 | Ezrin | Adaptors & Ty | 80 | 85 | 1 | - | 1.46 | 1.34 | 1.40 | 1.40 |
| 5501 | F69620 | C | 24 | FIN13 | Protein Phosp | 75 | 68 | 0 | - | 1.52 | 1.71 | 1.40 | 1.85 |
| 6501 | G55620 | A | 21 | GDNFR-alpha | Neuroscience | 45_60 | 62 | 1 | - | 1.49 | 1.90 | 2.20 | 1.29 |
| 2502 | H62120 | D | 7 | hILP/XIAP | Apoptosis | 57 | 58 | 2 | - | 1.59 | 1.54 | 1.39 | 1.75 |
| 2606 | P33420 | E | 10 | Prenylcysteine Lyase | Mitochondria/C | 58 | 59 | 2 | - | 1.36 | 1.92 | 2.00 | 1.31 |
| 6201 | R22020 | B | 29 | Rap1 | GTPases GTP | 21 | 21 | 2 | + | 1.50 | 1.31 | 1.46 | 1.35 |
| 2701 | R32820 | E | 9 | Rin1-70KD? | GTPases GTP | 90 | 70 | 2 | - | 2.97 | 1.94 | 1.42 | 4.05 |
| 1604 | T10120 | C | 6 | TAP | Nucleus & Nuc | 70 | 73 | 0 | - | 1.42 | 2.08 | 1.66 | 1.77 |
| 7301 | T87920 | B | 31 | TRP32-32KD | Apoptosis Cell | 32 | 32 | 0 | - | 1.63 | 1.33 | 1.38 | 1.57 |
| 202 | V47020 | C | 2 | VHR | Protein Phosp | 20 | 21 | 1 | - | 1.25 | 1.75 | 1.72 | 1.28 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Level 2a - low signal data, changes 1.5 to 1.9-fold in 4 of 4 comparisons** | | | | | | | | | | | | | |
| 902 | A59420 | A | 1 | Amphiphysin | Neuroscience | 125 | 138 | 4 | - | 4.89 | 1.55 | 1.99 | 3.81 |
| 4502 | A61220 | C | 21 | Annexin XI | Calcium Signa | 56 | 55 | 4 | + | 1.77 | 7.29 | 1.60 | 8.10 |
| 1502 | C67520 | C | 6 | CART1 | Cancer Resea | 53 | 57 | 4 | - | 2.86 | 1.46 | 2.15 | 1.95 |
| 501 | C39520 | C | 1 | CoRest | Nucleus & Nu | 66 | 63 | 4 | - | 4.40 | 2.30 | 6.87 | 1.47 |
| 1903 | C39220 | C | 7 | CTCF-137KD | Cancer Resea | 140 | 137 | 4 | - | 5.15 | 1.48 | 4.35 | 1.75 |
| 3604 | D63020 | C | 11 | Dystrobrevin | Neuroscience | 87 | 91 | 4 | - | 4.29 | 1.77 | 3.15 | 2.41 |
| 3203 | E78920 | C | 11 | ERAB-22.8KD | Neuroscience | 27 | 23 | 4 | + | 1.63 | 2.11 | 1.67 | 2.05 |
| 403 | M24520 | D | 1 | MEK2 | MAP Kinase P | 46 | 47 | 3 | - | 2.45 | 1.89 | 2.60 | 1.78 |
| 5703 | M92920 | D | 20 | Mre11 | Cell Biology | 81 | 90 | 4 | + | 1.55 | 10.50 | 1.50 | 10.90 |
| 3401 | N74120 | D | 11 | Na,K ATPase beta 3-34KD? | Neuroscience | 42 | 34 | 4 | - | 2.23 | 1.74 | 2.28 | 1.70 |
| 4401 | P21520 | B | 17 | Phospho-p38MAPK (T180/Y182) | MAP Kinase P | 42 | 39 | 3 | - | 1.79 | 1.62 | 1.97 | 1.47 |
| 2807 | R32820 | E | 9 | Rin1-120KD? | GTPases GTP | 90 | 120 | 4 | - | 2.64 | 2.08 | 1.78 | 3.10 |
| 3901 | R56420 | C | 10 | Sec8-139KD? | Membrane Res | 110 | 139 | 3 | + | 1.60 | 1.95 | 1.94 | 1.61 |
| 4302 | T92520 | C | 19 | TRAX-33KD | Nuclear Trans | 33 | 33 | 3 | + | 1.57 | 2.53 | 1.86 | 2.13 |
| 6603 | T21820 | D | 23 | TRP-1-80KD? | Cancer Resea | 70 | 80 | 4 | - | 1.73 | 1.90 | 1.62 | 2.03 |
| **Level 2b - low signal data, changes 1.25 to 1.5 fold in 4 of 4 comparisons** | | | | | | | | | | | | | |
| 4707 | F20120 | D | 12 | 4.1N-98KD | Nucleus & Nu | 135/100 | 98 | 4 | - | 1.26 | 1.72 | 1.50 | 1.44 |
| 8301 | A73820 | A | 34 | Acetylcholine Receptor alpha-42KD | Neuroscience | 49 | 41 | 4 | - | 2.17 | 1.44 | 2.13 | 1.47 |
| 7901 | A12520 | B | 30 | AIP1 | Apoptosis Cal | 105 | 99 | 3 | - | 1.34 | 1.40 | 1.30 | 1.44 |
| 5801 | C13620 | E | 19 | Caveolin 1-81KD | Adaptors & Ty | 22 | 81 | 4 | + | 22.43 | 1.45 | 25.32 | 1.29 |
| 2302 | C18520 | C | 9 | Cdk2 | Kinases Cell C | 33 | 33 | 4 | - | 2.16 | 1.36 | 2.26 | 1.30 |
| 1301 | C20820 | E | 4 | E-Cadherin-21KD? | Cell Adhesion | 120 | 21 | 4 | + | 1.27 | 1.96 | 1.41 | 1.77 |
| 6505 | E77020 | C | 33 | eIF-5 | Cancer Resear | 49 | 55 | 3 | - | 1.29 | 1.68 | 1.56 | 1.40 |
| 6601 | F82720 | C | 28 | FLAP | Cytoskeleton | 85 | 78 | 3 | + | 1.56 | 2.22 | 2.50 | 1.39 |
| 8507 | L89820 | E | 35 | LCB1 | Membrane Res | 53 | 52 | 3 | + | 1.53 | 1.37 | 1.56 | 1.34 |
| 2501 | L05620 | D | 6 | Lyn | Tyrosine Kinas | 56 | 55 | 4 | - | 1.87 | 2.28 | 1.27 | 3.36 |
| 5903 | P66520 | C | 24 | p140mDia-127KD? | GTPases Cyto | 140 | 127 | 4 | - | 2.03 | 2.05 | 2.91 | 1.44 |
| 7611 | S12220 | D | 27 | Phospho-Stat5 (Y694)-82KD? | Phosphotyrosi | 92 | 82 | 4 | - | 2.62 | 1.34 | 1.77 | 1.98 |
| 1908 | G12920 | D | 3 | Ras-GAP | Adaptors & Ty | 120 | 110 | 4 | - | 2.46 | 2.13 | 1.44 | 3.62 |
| 4604 | Y24420 | C | 21 | YAP | Adaptors & Ty | 74 | 76 | 4 | + | 2.21 | 1.42 | 1.66 | 1.89 |
| **Level 1a - changes greater than 2 fold in 3 of 4 comparisons** | | | | | | | | | | | | | |
| 6401 | A47520 | D | 22 | beta-Arrestin1-50KD | Neuroscience | 55 | 50 | 2 | + | 1.88 | 1.93 | | 2.96 |
| 7201 | M99920 | C | 34 | MnSOD | Nitric Oxide M | 25 | 21 | 0 | + | 2.08 | 2.11 | 3.94 | |
| 3102 | U66820 | B | 8 | UbcH7 | Cell Cycle | 18 | 19 | 1 | - | 2.29 | 1.93 | 4.71 | |
| **Level 1b - changes 1.5-1.9 fold in 3 of 4 comparisons** | | | | | | | | | | | | | |
| 3605 | A30120 | C | 10 | Annexin VI-71KD | Calcium Signa | 70 | 71 | 1 | - | | 3.63 | 1.99 | 1.70 |
| 3003 | A63220 | A | 11 | ARF-3 | GTPases Neur | 20 | 18 | 0 | + | 1.47 | 1.50 | | 2.10 |
| 7201 | B36420 | B | 30 | Bad | Apoptosis Car | 23 | 21 | 1 | - | 1.54 | 1.46 | (+)1.68 | 3.75 |
| 2201 | C70820 | C | 9 | CDC42 | Cancer Resea | 22 | 21 | 1 | + | 1.57 | 1.55 | 3.07 | (-)1.26 |
| 2604 | C62920 | C | 9 | CUL-2 | Cell Cycle Car | 76 | 71 | 0 | - | | 2.24 | 1.52 | 1.51 |
| 5403 | M37520 | D | 20 | ERK1-42KD | MAP Kinase P | 44/42 | 42 | 2 | | | 4.66 | 1.49 | 3.69 |
| 5401 | F37720 | B | 24 | Fas Ligand-34KD | Apoptosis Imr | 37 | 34 | 0 | + | 1.47 | 1.59 | 3.03 | (-)1.29 |
| 2201 | N25720 | B | 3 | Nm23 | Cancer Resea | 17 | 20 | 0 | - | 1.75 | 1.68 | 2.36 | |
| 7802 | N14920 | E | 32 | NSF | Neuroscience | 82 | 82 | 1 | + | 2.00 | 1.65 | | 2.71 |
| 3601 | N43620 | B | 8 | Nucleoporin p62 | Nuclear Trans | 62 | 63 | 1 | - | 1.78 | 1.77 | 2.79 | |
| 5101 | R24720 | C | 23 | RBP | Cancer Resea | 21 | 20 | 1 | + | 1.66 | 2.03 | 8.22 | (-)2.44 |
| 4101 | M82020 | C | 15 | Stathmin/Metablastin-17KD? | Cytoskeleton C | 19 | 17 | 1 | - | (+)1.53 | 5.72 | 1.68 | 2.22 |
| 7101 | U85520 | C | 37 | UbcH6 | Cell Cycle Apo | 21 | 18 | 1 | + | 2.10 | 1.81 | 3.06 | |

| Level 0 - No significant protein changes | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7801 | F58420 | B | 31 | 5-Lipoxygenase | Adaptors & Ty | 79 | 77 | 2 | | | | | |
| 4601 | A84320 | B | 10 | ALDH | Cell Biology C | 55 | 56 | 0 | | | | | |
| 1906 | C45520 | B | 2 | Calnexin-95KD | Calcium Signa | 90 | 95 | 0 | | | | | |
| 1002 | C73220 | A | 2 | CRP1 | Cytoskeleton | 23 | 20 | 4 | | | | | |
| 4702 | 556597 | D | 14 | DNA Topo I | 0 | 100 | 92 | 1 | | | | | |
| 6501 | P61520 | C | 32 | Dok1/p62dok-71KD? | Adaptors & Ty | 62 | 71 | 0 | | | | | |
| 1402 | C20820 | E | 4 | E-Cadherin-31KD? | Cell Adhesion | 120 | 31 | 3 | | | | | |
| 4104 | E15920 | C | 21 | eIF-5a-21KD | Nucleus & Nu | 18 | 21 | 3 | | | | | |
| 3401 | F65020 | C | 13 | Flotillin-1-45KD | Caveolae Cell | 48 | 45 | 4 | | | | | |
| 3301 | G29620 | B | 8 | G beta-30KD | GTPases GTP | 37 | 30 | 4 | | | | | |
| 3306 | G76220 | D | 10 | GS27 | Membrane Res | 27 | 24 | 0 | | | | | |
| 4501 | H86320 | C | 15 | hPrp17 | Cell Biology | 66 | 57 | 2 | | | | | |
| 5402 | I95020 | D | 18 | I kappa B epsilon-42KD | Transcription | 45 | 42 | 4 | | | | | |
| 2402 | I58620 | B | 6 | Inhibitor 2 | MAP Kinase P | 32 | 37 | 2 | | | | | |
| 6701 | M14520 | D | 24 | MCM4-90KD? | Nucleus & Nuc | 100 | 90 | 3 | | | | | |
| 4501 | M77320 | D | 12 | MST3 | Apoptosis MAP | 52 | 55 | 2 | | | | | |
| 5401 | E17120 | C | 22 | pan ERK-40KD | MAP Kinase P | 42_85 | 40 | 0 | | | | | |